Immunity to MHC Class I Antigen after Direct DNA Transfer into Skeletal Muscle[1]

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Plasmid cDNA encoding the α-chain of either membrane-bound (pcRT.45) or secreted (pcRQ.B3) RT1A^a MHC class I Ag were transferred to Lewis (RT1^l) rat skeletal muscle by direct injection. Rats were challenged 7 days later with an ACI (RT1^a) heterotropic heart transplant, and cardiac allograft survival, RT1A^a-specific antibody levels, and frequency of ACI-specific CTL were monitored. Graft rejection was accelerated by ≥2 days in an Ag-specific and dose-dependent manner in pcRT.45-injected rats. The pcRQ.B3-injected rats also rejected grafts more rapidly; however, graft rejection was accelerated by only 1 day, and graft infiltrates were less pronounced than in pcRT.45-injected rats. Injection of pcRT.45 resulted in an increase in ACI-specific CTL precursor frequency 3 days post-transplant, whereas there was no significant change in rats pretreated with pcRQ.B3 injection. Compared with rats injected with a control plasmid encoding firefly luciferase, transfer of pcRT.45 resulted in an increase in RT1A^a-specific IgG and IgM antibody 3 days after heart transplantation. Transfer of pcRQ.B3 resulted in a similar mean increase in RT1A^a-specific IgG and IgM antibody after transplantation, but the variability from rat to rat was greater, with some animals exhibiting strong priming, and others showing little or no priming by gene injection. Our results suggest that skeletal muscle can express either membrane-bound or secreted MHC class I Ag after gene transfer, but that the membrane-bound form is more immunogenic than the secreted form in the high responder Lewis rat. Direct DNA transfer to skeletal muscle provides a rapid and specific approach to studying immunity to allogeneic MHC Ag. *Journal of Immunology,* 1994, 152: 413.

The Ir[3] to alloantigen depends fundamentally on the form and mode of presentation of the Ag. In transplantation, MHC class I Ag may play an important role by priming the Ir resulting in accelerated rejection of allografts, or by inducing immunologic unresponsiveness (1–3). Donor MHC class I Ag may be released from an allograft in at least two basic forms: 1) as membrane-associated Ag either on cell surfaces or shed from cell surfaces into the extracellular space; or 2) as water-soluble secreted Ag. What determines whether these different forms of MHC class I molecules are immunogenic, or have tolerogenic properties in vivo is largely unknown. Some evidence suggests that purified water-soluble MHC class I Ag may be less immunogenic (4) and less tolerogenic than membrane-bound Ag (5), whereas other data suggest that soluble forms of MHC class I (6, 7) and other Ag (8) may have a higher potential for inducing immunologic unresponsiveness with minimal risk for sensitization. However, the difficulty of obtaining purified lipid- and water-soluble MHC molecules in large quantities has precluded extensive studies to compare their immunologic effects. In addition, it is difficult to mimic the activities of cell-derived graft Ag that are secreted, or shed continuously, using protein preparations injected daily or via continuous infusion methods. In the present study we have devised a strategy to study the immunologic effects of the different forms of MHC class I Ag in vivo using direct gene transfer to skeletal muscle. With this method an allogeneic donor strain rat MHC class I Ag (RT1A^a) was expressed in recipient Lewis rat (RT1A^l) myocytes before transplantation. This high responder rat strain was chosen because membrane-bound RT1A^a Ag is known to be strongly immunogenic in the RT1A^l haplotype (9). Therefore, if secreted RT1A^a had different immunologic properties, they

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[3] Abbreviations used in this paper: Ir, immune response; L-M(TK), L-M mouse fibroblast cells; PBS/BSA, PBS + 0.5% BSA; PCR, polymerase chain reaction.

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could easily be distinguished from those of the strongly immunogenic membrane-bound RT1A^a.

The method of direct gene transfer to skeletal muscle has been used to express a variety of gene products in a stable manner, including firefly luciferase and β-galactosidase (10–12). Although rodent myocytes normally express MHC class I Ag at a low level, they are capable of expressing high levels of the Ag after interferon treatment (13). Thus, MHC class I Ag expression in myocytes does not appear to be limited by such factors as β_2-microglobulin insufficiency or lack of peptide-loading capacity that might prevent class I assembly after α-chain cDNA transfer. One advantage of using direct cDNA transfer is that the cDNA for an allogeneic MHC class I molecule can be modified, and the immunologic effect of the alteration may readily be determined in vivo. Recently, foreign proteins have been expressed by muscle cells after direct gene injection, and typical T and B cell immunologic responses are generated (14, 15). Direct transfer of cDNA also permits the expression of a single Ag, avoiding the problem of contaminating substances in purified Ag preparations. In addition, myocyte syncytia are not mobile, ruling out the migration of genetically altered cells to central lymphoid tissue. Other gene transfer modalities have been useful in expressing foreign MHC genes in vivo. In some studies tumor cells and hemopoietic tissues have been transfected with MHC genes in vitro using DNA-calcium phosphate co-precipitation or retroviruses, followed by in vivo cell transfer (16, 17). Although these methods are useful for transfecting a wider variety of cells and tissues, direct gene transfer to skeletal muscle has advantages including: 1) it is relatively simple, 2) it does not require in vitro transfections before in vivo transfer (although, viral vectors can be directly transferred in vivo), 3) it is noninfectious, and 4) it avoids the risk of an Ir to a viral vector that could interfere with the interpretation of the Ir against the Ag of interest.

In this study, the cDNA encoding a rat membrane-bound MHC class I molecule RT1A^a was altered to encode a secreted, water-soluble, form of RT1A^a. Plasmid constructs encoding membrane-bound and secreted RT1A^a were expressed in myocytes after direct cDNA transfer to skeletal muscle, allowing us to compare the humoral, CTL, and graft rejection responses to membrane-bound vs secreted MHC class I Ag in vivo. Despite the ability of transferred cDNA encoding either membrane-bound or secreted MHC class I Ag to prime an antibody response to RT1A^a, our results indicate that membrane-bound MHC class I Ag is more effective than secreted MHC class I Ag at priming ACI-specific CTL and causing accelerated rejection of ACI cardiac allografts. Gene transfer provides a novel method for modifying the allograft response in a transplantation model, and also provides a useful technique for studying the Ir to different forms of allogeneic MHC class I Ag.

Materials and Methods

DNA constructs

The plasmid containing the RT1A^a cDNA, pBS3.3/1 (18, 19), was provided by Dr. Jonathan Howard (Babraham Institute, Cambridge, UK). The 1.5-kb *Eco*RI fragment of pBS3.3/1 was isolated and subcloned into the CMV expression vector pcDNA1 (In Vitrogen, San Diego, CA) to create the pcRT.45 expression construct (see Fig. 1*A*). The pCMVLux construct encoding firefly luciferase was obtained from Dr. Jon Wolff (Waisman Center, University of Wisconsin, Madison, WI).

A recombinant construct encoding secreted RT1A^a was developed by combining a portion of the α3 region of the RT1A^a cDNA with the transmembrane region of the secreted mouse MHC class I-like molecule, Q10^b (20) using rPCR methods (21). The upstream primer A (5′-ACATGGAGCTTGTGGAGACC-3′) and downstream primer B (5′-TCAGTGGAAGGAGGAGGTTCCCATCTCTGGGAAA-3′) define a 150-bp PCR fragment starting just upstream of the *Pst*I site in the middle of the α3 domain of the RT1A^a molecule to the junction of the α3 and transmembrane domain. A 130-bp PCR fragment of the transmembrane domain of Q10^b was defined by the upstream primer C (5′-GAACCTC-CTCCTTCCACTGA-3′) and the downstream primer D (5′-GTC-GACTCTAGATTTCCCACCCGTGTTTCTCCTTCT-3′) which contains an *Xba*I site. Because a portion of primer B is complementary to primer C, denaturation and annealing of the two PCR products creates a new fusion product that is amplified using the two outside primers A and D in a third PCR reaction. After restriction enzyme digestion, this fusion product was ligated in the *Pst*I and *Xba*I sites of the pcRT.45 plasmid to create the final construct encoding secreted RT1A^a, pcRQ.B3 (see Fig. 1*B*). dsDNA sequencing of the plasmid using Sequenase (U. S. Biochemical, Cleveland, OH) demonstrated the expected sequence in frame.

Transient transfection

L-M(TK) cells, American Type Culture Collection, Rockville, MD, were transfected with either pcRT.45 or pcRQ.B3 using Lipofectin (GIBCO BRL, Gaithersburg, MD). After an overnight incubation with serum-free OPTI-MEM (GIBCO BRL) at 37°C, 5% CO_2, FCS was added to each plate at a final concentration of 10%. Cells and media were harvested on day 3 to test for cell-surface expression by flow cytometry and secreted RT1A^a production by ELISA, respectively. For flow cytometry, cells were first reacted with the RT1A^a-specific antibody R3/13 (Bioproducts for Science, Indianapolis, IN), washed with PBS, then reacted with goat anti-rat IgG-FITC (Jackson ImmunoResearch, West Grove, PA). After two PBS washes, flow cytometry was performed with a Coulter Profile (Coulter Immunology, Hialeah, FL).

Primary cultured Lewis rat myoblasts were prepared (as described in detail elsewhere) (22) and transfected with pcRT.45, pcRQ.B3, or pCMVLux using the Lipofectin method. Total RT1A^a protein was measured by ELISA in both culture supernatants and cell lysates.

ELISA for RT1A^a

The ELISA for RT1A^a was performed on 96-well half-area EIA plates (Costar Corp., Cambridge, MA). Wells were coated with 10 μg/ml MRC OX-18 (specific for a monomorphic determinant of RT1A; Bioproducts for Science) overnight at 4°C, blocked for 1 h with 2% BSA in PBS, and washed. Purified RT1A^a standard was prepared from detergent lysates of ACI spleens by MHC class I affinity chromatography as described by DeVito et al. (23), except that Sepharose 4B (Sigma, St. Louis, MO) coupled to OX-18 and RT1A^a-specific mAb 211-4D9 (hybridoma provided by Heinz Kunz, University of Pittsburgh, Pittsburgh, PA) was used as the final affinity column matrix. Test samples or affinity-purified RT1A^a Ag were titered in the wells, and incubated 4 to 12 h at 4°C. After washing, a 1/20 dilution of R3/13 or isotype-control antibody was added, and incubated 1 h at room temperature. The plate was washed, and a 1/200 dilution of mouse anti-rat IgG2b-biotin (Bioproducts for Science) was added. After a 1-h incubation at room temperature the plate was washed, and a 1/5000 dilution of streptavidin horseradish peroxidase (Sigma) was added for 30 min at room temperature. The plate was washed, and *o*-phenylenediamine was added to allow enzymatic conversion of the substrate to a chromogenic product measured at 490 nm (Bio-tek microplate autoreader, Bio-tek Instruments, Burlington, VT). RT1A^a concentration was quantitated by linear regression analysis using Bio-tek

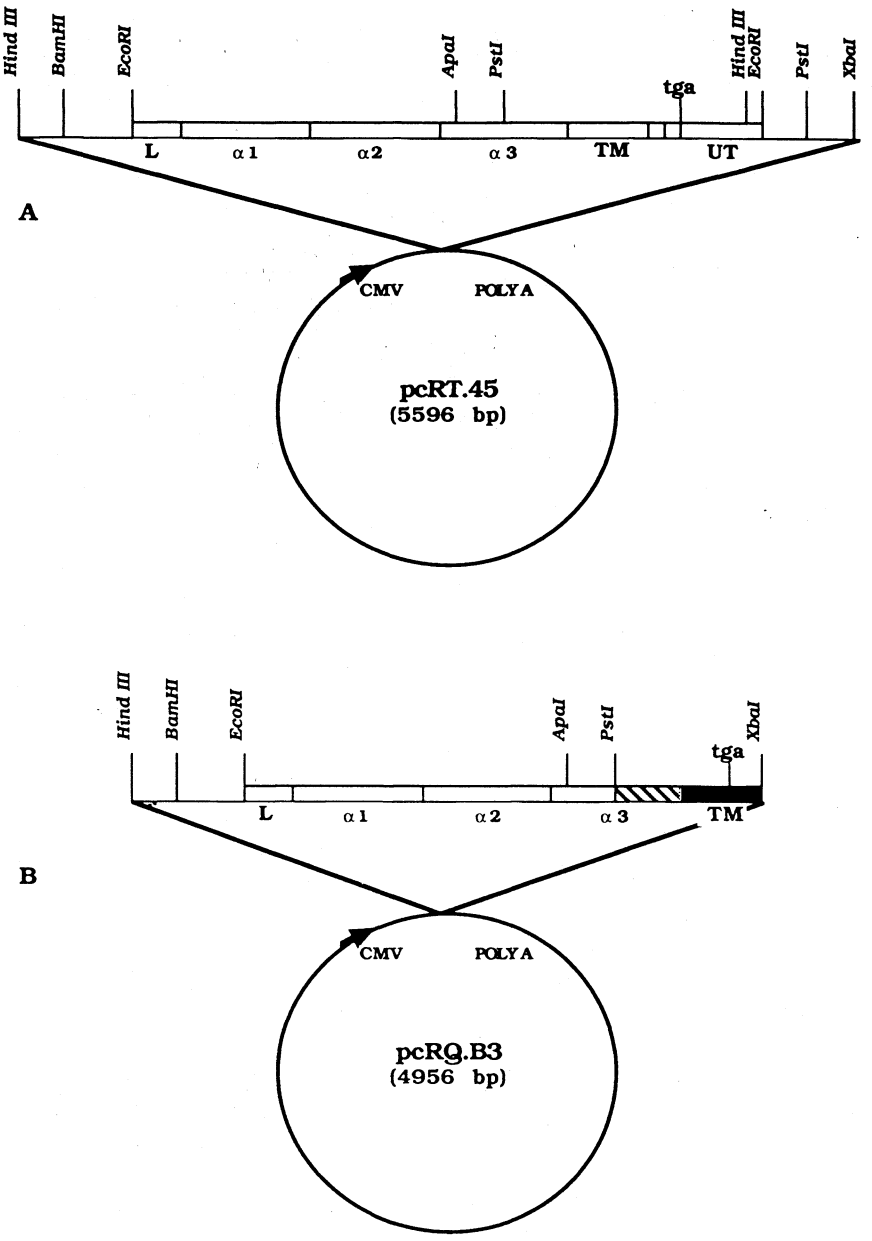

FIGURE 1. Plasmid maps for *A)* pcRT.45 (encoding membrane-bound RT1A^a), and *B)* pcRQ.B3 (encoding secreted RT1A^a) are shown, as described in *Materials and Methods*. The RT1A^a (*cross-hatched*)/Q10^b (*solid*) junction created by rPCR is shown, as is the approximate location of the first stop codon in the open reading frame. *L*, leader sequence; *TM*, transmembrane; *UT*, 3′ untranslated region.

software, and the purified RT1A^a standard. This assay was found to be sensitive to 0.5 ng/ml of RT1A^a for tissue culture supernatants and cell lysates. The assay was less sensitive (15 ng/ml) when testing for RT1A^a in serum because of higher background OD readings.

In vivo gene transfer and cardiac transplantation

In vivo gene transfer was performed by direct muscle injection of the quadriceps muscle, as described previously (10). Heterotopic cardiac transplants were performed using a modification of the technique originally described by Ono and Lindsey (24). Donor hearts were grafted in the recipient rat's abdomen by anastomosing donor and recipient aorta, and donor pulmonary artery to the recipient's inferior vena cava. Grafts were palpated to assess rejection, and nonpalpable contractions prompted examination by direct inspection by laparotomy. Graft rejection time was defined as the time at which no cardiac contractions were either palpable or visible by direct inspection at laparotomy. Grafts were checked twice daily and time to rejection was measured from the day of transplant to the day of rejection in whole day increments.

Histochemistry

Morphologic changes in transplanted hearts were evaluated by staining paraffin sections with Mayer's hematoxylin and eosin. For mononuclear cell enumeration, stained sections were viewed with a video camera attached to a microscope, and images were processed with the microcomputer-based image analysis system, Image-1 (Universal Imaging Corporation, West Chester, PA). Fifteen areas from multiple sections of the myocardium were randomly selected and digitized for each heart allograft. To determine the amount of mononuclear cell infiltration, the number of dark staining nuclei per area was determined using the measure object function of the software after user input of the threshold object pixel value. The mean number ± SD of mononuclear cells per 15 areas was reported.

The quadriceps muscle was removed and frozen for immunohistochemical examination at the time of rejection of the transplanted heart. Immunofluorescent staining was performed on 6 μm cryostat sections of rat skeletal muscle fixed with 2% paraformaldehyde. After washing with PBS + 0.05% Tween 20, sections were incubated overnight at 4°C with

R2-15S rat anti-RT1A^a mAb (Bioproducts for Science). After washing in PBS and incubating with PBS + 0.05% Tween 20 + 3% goat serum, slides were incubated with biotinylated mouse anti-rat IgG2a (Bioproducts for Science) for 2 h at room temperature. After rinsing with PBS, streptavidin-Texas Red conjugate was added. Control sections were stained with IgG2a rat anti-RT1A^u mAb (Bioproducts for Science) as the primary antibody.

RIA for MHC class I-specific alloantibodies

Anti-ACI class I IgM and IgG antibody levels were measured using a modification of the method reported by Morris and Williams (25). Briefly, rat serum was diluted 1/10 in PBS/BSA, and 25 μl were added to duplicate 12 × 75 mm polystyrene tubes. ACI and Lewis (control) strain blood was collected in heparinized tubes, washed two times with PBS/BSA, and RBC were diluted to 10% (v/v). PVG (control-RT1A^{c}B^{c}D^{c}C^c) and PVG.R1 (RT1A^{a}B^{c}D^{c}C^c) RBC were used in some experiments. Diluted cells were added to each tube in 25-μl aliquots, and the serum/cell mixture was incubated for 1 h at room temperature. Cells were washed two times with PBS/BSA, resuspended in 100 μl of PBS/BSA, and incubated 1 h with either ^{125}I-labeled goat anti-rat IgM or ^{125}I-labeled goat anti-rat IgG (antibodies purchased from Jackson ImmunoResearch) antibody at 300,000 cpm/tube. Antibodies were radiolabeled with [^{125}I]Na (Amersham, Arlington Heights, IL) using the IODO-GEN iodinating reagent as specified by the manufacturer (Pierce, Rockford, IL). After incubation, cells were washed as described above, resuspended in PBS/BSA, transferred to fresh tubes, and cpm was determined. Statistical differences in cpm between experimental and control groups were determined by a two-tailed *t*-test comparing independent sample means.

Limiting dilution analysis

Limiting dilution analysis for CTL precursor frequency was performed as described in detail previously (26). Twelve replicate cultures of responder cells at six dilutions were used in the assay. Target cells were either Con A-stimulated ACI or Wistar-Furth (RT1^u-third party) splenocytes. The CTL precursor frequency was calculated using the maximum likelihood method as outlined by Derry and Miller (27).

Results

To determine if the plasmid constructs encoding membrane-bound and secreted RT1A^a (Fig. 1) were functional, mouse L-M(TK) cells were transfected with either pcRT.45 or pcRQ.B3 in a transient assay. Figure 2 shows an example of one of three experiments with different preparations of plasmids. The flow cytometry analysis indicated that pcRT.45, but not pcRQ.B3 cDNA-transfer, results in cell-surface expression of RT1A^a 3 days post-transfection. Approximately 25% of the pcRT.45-transfected cells expressed the cDNA product at a broad range of intensities. Culture supernatants from the same transfections were analyzed for secreted RT1A^a by an ELISA. Secreted RT1A^a was not detected in the culture supernatants from the transfection performed with pcRT.45 plasmid. However, transfection of the pcRQ.B3 plasmid resulted in 6 ng/ml of RT1A^a in the culture supernatant (the mean ± SD in three separate experiments was 6 ± 3 ng/ml). Because transfection efficiency was approximately 25% and the total number of cells plated was 1×10^6 in 1.5 ml of culture medium, the total amount of secreted RT1A^a measured was 9 ng/2.5×10^5 cells. To more closely simulate MHC class I expression in donor skeletal muscle, total RT1A^a production was assessed in primary cultured Lewis myoblasts transfected with pcRT.45 or pcRQ.B3 (Table I). Cell lysates and culture supernatants

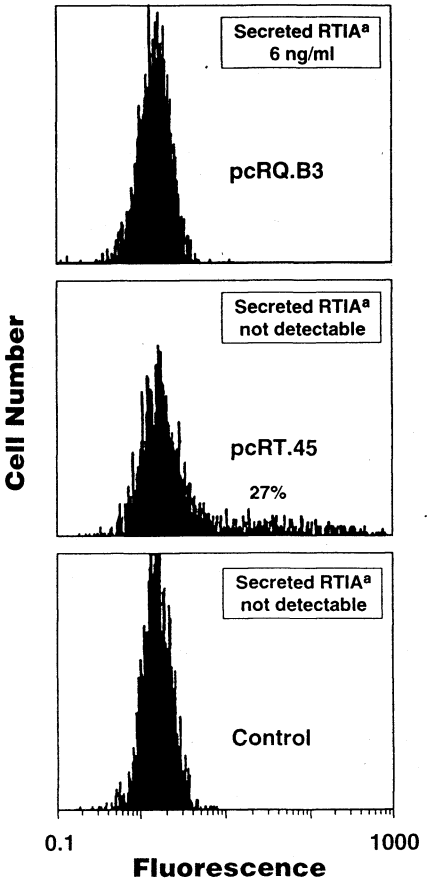

FIGURE 2. Cell-surface expression of RT1A^a on mouse L-M(TK) fibroblast cells after transient transfection with pcRQ.B3 (*upper*) and pcRT.45 (*middle*), compared with cells transfected without addition of plasmid (*lower*). Results from one representative experiment are shown. With pcRT.45 transfection, 27% of the cells were positive for surface RT1A^a by FACS; RT1A^a-positive cells were not detectable with pcRQ.B3 transfection. The *inset* in each histogram shows the amount of secreted RT1A^a detected by ELISA in the culture supernatant from the same transfection.

were measured over a 3-day period for RT1A^a by ELISA. The pcRT.45-transfected myoblast cell lysates and pcRQ.B3-transfected cell culture supernatants showed similar amounts of RT1A^a at 48 and 72 h. Secreted RT1A^a was not detectable in culture supernatants from pcRT.45-transfected cells at any of the time points tested. Cell lysates from pcRQ.B3-transfected cells demonstrated no detectable RT1A^a at 24 or 48 h post-transfection, but 2 ng of RT1A^a was present in the lysate at 72 h.

Expression of RT1A^a was examined 7 days after injection of pcRT.45. Immunofluorescent staining with mAb specific for RT1A^a demonstrated that Lewis muscle cells at the injection site expressed RT1A^a Ag on their cell surface (Fig. 3*A*). Muscle cells distant from the injection site did not express RT1A^a protein. This pattern of myocyte expression of transfected plasmid DNA is similar to that previously reported for the reporter gene Lac Z (12). As a

Table I. *Total RT1A*[a] produced by pcRT.45 and pcRQ.B3-transfected Lewis myoblasts[a]

Plasmid	Culture Period (h)	Lysate (ng/2 × 10^5 cells)	Supernatant (ng/2 × 10^5 cells)
pcRT.45	24	4	<0.5[b]
	48	7	<0.5
	72	17	<0.5
pcRQ.B3	24	<0.5	<0.5
	48	<0.5	13
	72	2	23

[a] Results are representative of one of two experiments performed, and values shown are the mean of duplicate determinations. Lysates and culture supernatants from myoblasts transfected with pCMVLux did not show OD readings above control at any of the time points tested.
[b] Below the sensitivity of the assay.

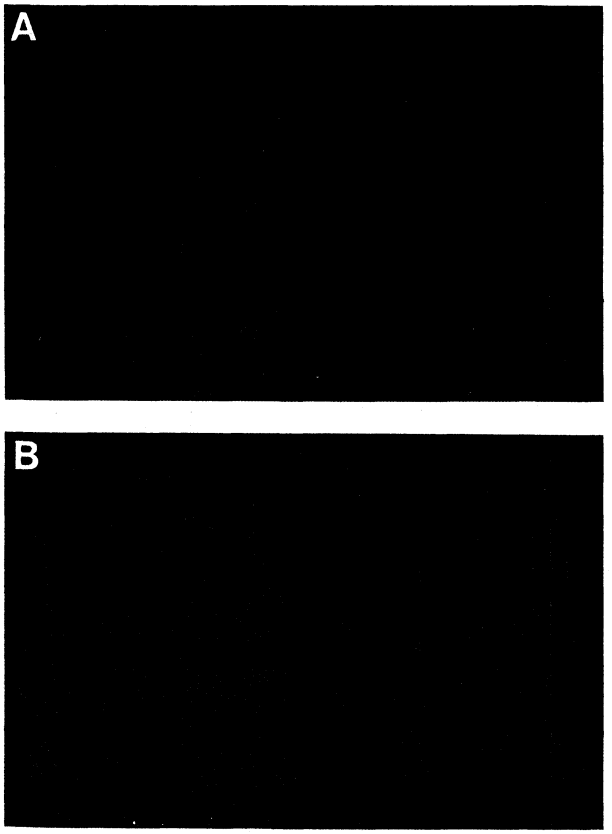

FIGURE 3. *A*, immunofluorescent staining of pcRT.45-injected rat quadriceps muscle shown here in transverse cross-section shows RT1A[a] expression on the cell membrane of transfected myocytes (×500). *B*, transverse section of pcRT.45-injected Lewis rat quadriceps muscle stained with mAb specific for RT1A[u] shows only background fluorescence in contrast to *A*.

control, the adjacent section was stained with an isotype-matched mAb specific for RT1A[u], and only background fluorescence was detected (Fig. 3*B*). Lewis muscle injected with pCMVLux also did not stain with either anti-RT1A[a] or anti-RT1A[u] mAb (data not shown).

Table II. *Survival of heterotopic cardiac allografts after cDNA transfer*

Donor	Plasmid	Dose	Graft Survival (days)	p Value[a]
ACI	None		6,6,6,6,6,7	control
ACI	pcRT.45	1,000 μg	3,4,4,4,4,4	<0.001
		600 μg	4,4	
		400 μg	4,4	
		300 μg	4,5	
		100 μg	5,5	
		50 μg	4,4	<0.001
		25 μg	4,5,5,6	
		10 μg	4,5,5	
		5 μg	5,5,5	
		1 μg	5,5,6	
		500 ng	6,6	
		100 ng	6,7	NS
ACI	pcRQ.B3	1,000 μg	5,5,5,5,5[b]	
		600 μg	5,5,6	0.003
		400 μg	5,5,6	
		200 μg	6	
ACI	pCMVLux	1,000 μg	6,6,6,6,6	
		600 μg	6,6	NS
WF	None		6,6,6,7,7,8,9,9	control
WF	pcRT.45	1,000 μg	7,8,8,9,9	
		600 μg	6,7,9	
		400 μg	6,9	
		200 μg	7,9	NS
		100 μg	8	
		50 μg	7,7	

[a] Vs control with the same donor (Mann-Whitney test).
[b] $P < 0.001$ when compared with the 1000-μg dose of pcRT.45.

One week after pcRT.45 plasmid transfer, an ACI heart was transplanted to the Lewis rat recipient. Cardiac transplants rather than skin or other tissues were used to measure the in vivo alloresponse because the time to rejection can be more objectively evaluated by monitoring the presence or absence of myocardial contraction by direct visual inspection. Table II summarizes the graft survival time of ACI to Lewis heterotopic cardiac allografts (complete MHC and non-MHC mismatch). Without DNA transfer, ACI hearts were consistently rejected at 6 days. Injection of as little as 1 μg of pcRT.45 resulted in accelerated rejection, and recipients receiving injection of 400 μg or more of pcRT.45 rejected at 4 days or less. The degree of the sensitization correlated with the dose of pcRT.45 administered.

To determine whether sensitization was specific for RT1A[a], Wistar-Furth (third party) hearts were transplanted to Lewis recipients after pcRT.45 injection (Table II). There was no alteration of graft survival with any dose of DNA administered compared with noninjected controls. In addition, injection of 600 to 1,000 μg of a control plasmid encoding firefly luciferase (pCMVLux) did not alter ACI-heart graft survival, although this protein is well expressed after direct injection into rodent myocytes (10, 11).

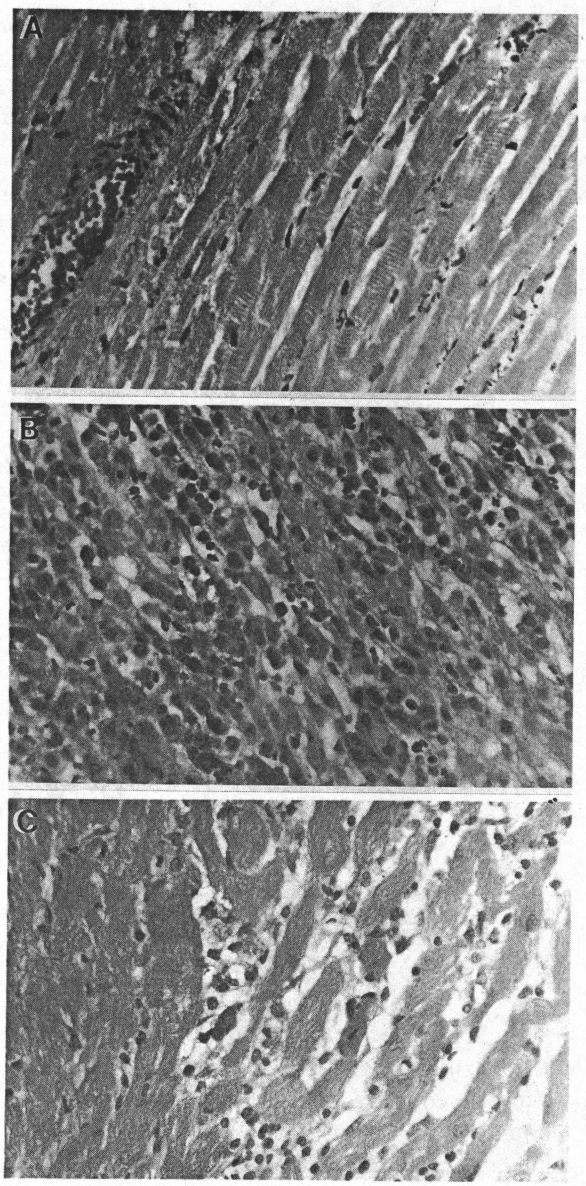

FIGURE 4. Micrographs of Mayer's hematoxylin-eosin stained heart allografts 3 days post-transplantation from pCMVLux (*A*), pcRT.45 (*B*), and pcRQ.B3 (*C*), -injected rats (×400).

Skeletal muscle injection of 1 mg of plasmid encoding secreted $RT1A^a$ (pcRQ.B3) 1 wk before heart transplantation accelerated rejection slightly, but consistently, from 6 to 5 days (Table II). A similar degree of acceleration was observed with a 200-fold lower dose of pcRT.45 (5 μg). Histologically, hearts from pcRQ.B3-injected rats showed a lesser degree of mononuclear cell infiltrate on day 3 post-transplant, compared with pcRT.45-injected rats (Fig. 4). Image-1 analysis of cardiac allografts on day 3 post-transplant confirmed a denser mononuclear cell infiltrate in hearts transplanted to pcRT.45-primed recipients compared with pcRQ.B3-injected recipients (6,500 ± 2,683 vs 1,567 ± 900 per 1 mm^2, respectively), and even less infiltrate in pCMVLux-injected controls (110 ± 75 per 1 mm^2). Regardless of the plasmid injected, all hearts showed tissue necrosis and a mononuclear cell infiltration typical of acute rejection at the time of rejection (data not shown).

To determine if specific priming of the humoral response to $RT1A^a$ had occurred by gene transfer, the specific antibody response was measured using a RIA. Figure 5 summarizes data from two experiments in which ACI-specific IgG and IgM antibody levels were determined after pcRT.45, pcRQ.B3, or pCMVLux injection and subsequent ACI cardiac transplantation. Seven days after injection, but before heart transplantation, a 1-mg dose of pcRT.45 did not result in an allospecific IgM or IgG antibody response. Sera were also collected 3 days post-transplantation to determine whether priming of the antibody response to $RT1A^a$ had been induced by gene transfer. When compared with the weak post-allograft anti-ACI IgM antibody response demonstrated by rats injected with pCMVLux control plasmid, the ACI-specific IgM antibody levels were consistently higher in rats pretreated with a 1-mg dose of pcRT.45. Although pcRQ.B3 injections elicited an ACI-specific IgM response in some animals, the response was found to be more variable. Acceleration of the post-allograft IgG antibody response also occurred after pcRT.45 or pcRQ.B3 injection, compared with rats injected with control plasmid. As with the IgM response, a more variable antibody (IgG) response was observed in both experiments after pcRQ.B3 gene transfer. More specifically, three out of six animals tested showed anti-$RT1A^a$ IgM and IgG antibody levels equal to, or greater than, the levels observed with pcRT.45 injection; the other three rats showed either relatively weak antibody responses, or antibody responses similar to control plasmid injected rats. The same rats that demonstrated a relatively high IgM antibody response, also showed a high IgG antibody response. The presence of $RT1A^a$-specific antibodies in the experiments described above was confirmed in a separate experiment; antibodies were found to bind PVG.R1 ($RT1A^aB^cD^cC^c$) RBC, but not PVG ($RT1A^cB^cD^cC^c$) RBC (data not shown).

CTL frequency determinations were performed to evaluate the response of CTL precursors to MHC class I cDNA-transfer (Table III). Spleens were obtained from plasmid-injected rats on day 3 post-transplant, and tested for frequency of ACI-specific CTL. Compared with control plasmid-injected rats, pcRT.45-injected rats showed a marked increase in frequency of CTL, suggesting priming by the cDNA-transfer. In contrast, the frequency of ACI-specific CTL was not significantly altered by pcRQ.B3 injection.

Discussion

Direct gene transfer to skeletal muscle in vivo permits the expression of a single foreign protein without potential impurities in the Ag or cell preparation caused by imperfect protein isolation techniques. The application of this technique

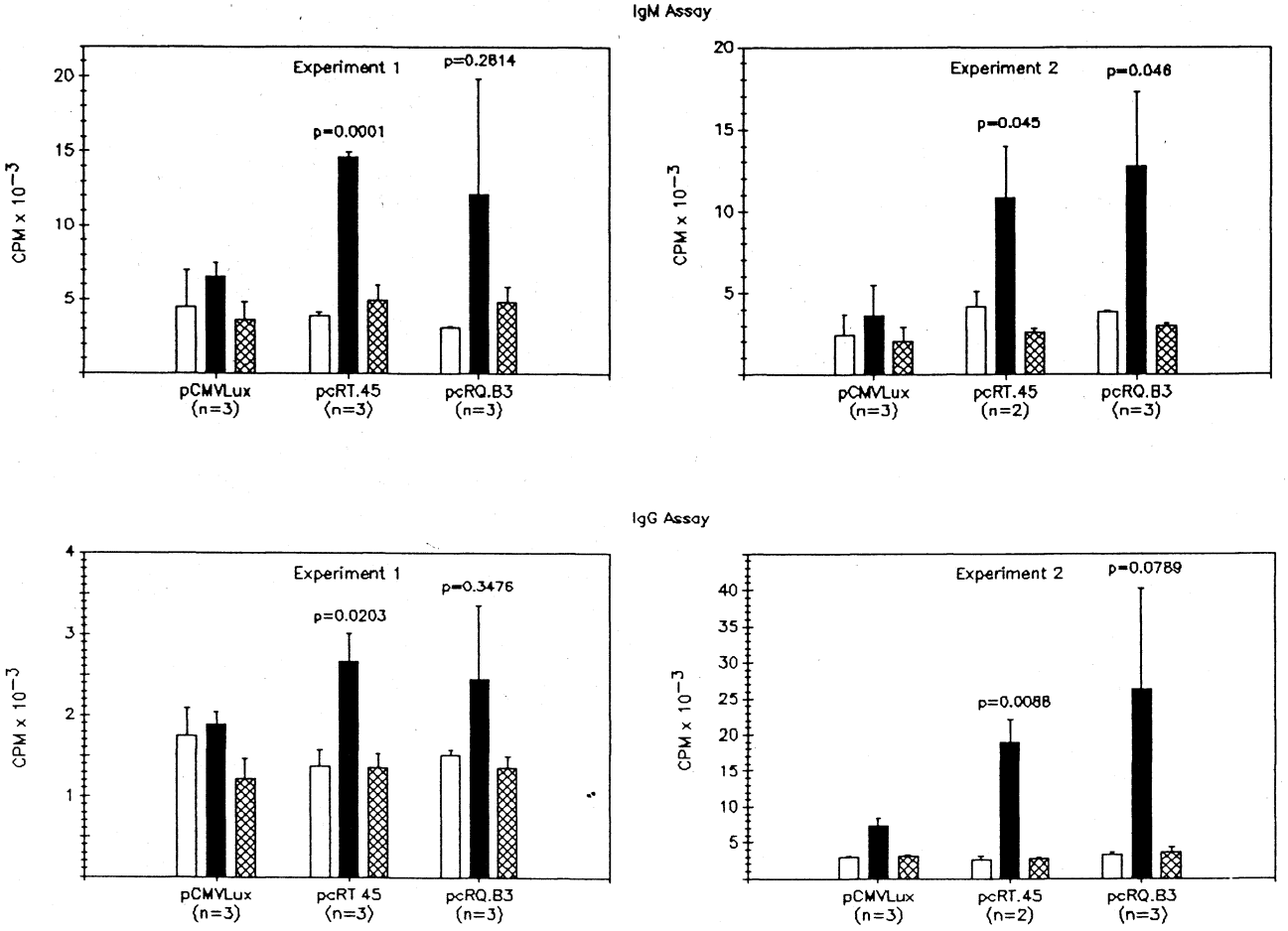

FIGURE 5. The effect of gene transfer on ACI-specific antibody production was tested by a RIA. Serum RBC-binding IgM and IgG antibody levels were measured: 1) 7 days after gene transfer to Lewis rats, but before ACI cardiac transplant, vs ACI RBC (*open bars*); 2) 3 days after ACI cardiac transplants vs ACI RBC (*closed bars*); or 3) 3 days after ACI cardiac transplants vs control Lewis RBC (*cross-hatched bars*). The *graphs at the top* represent data from each of two separate experiments where anti-ACI IgM antibody levels were measured; the *bottom graphs* are data from two separate experiments where anti-ACI IgG antibody levels were measured. Results represent the mean ± SD of pooled data from multiple rats. Statistical comparisons are relative to pCMVLux controls at the same time point (*t*-test).

Table III. *Effect of gene transfer on the frequency of ACI-specific CTL*

Plasmid	No.	1/Frequency	
		ACI targets	Wistar-Furth targets (third party)
pCMVLux (control)	3	3,476 ± 1,442	10,322 ± 999
pcRT.45	3	1,077 ± 378[a]	10,056 ± 5,902
pcRQ.B3	3	2,135 ± 452	9,484 ± 2,756

[a] $p < 0.05$ compared with control (*t*-test).

to transplantation offers the possibility of measuring the impact of constitutively expressed, genetically engineered, MHC proteins on graft rejection or prolongation. Expression by a nonmobile cell offers the analogy with a long term "passenger cell"-depleted graft, and precludes the element of recirculation of alloantigen-bearing cells.

Experiments presented in this study demonstrate that MHC cDNA transfer in vivo results in expression of allogeneic class I Ag by myocytes. Membrane-bound $RT1A^a$ was demonstrated by immunohistochemistry in pcRT.45-injected skeletal muscle 7 days postinjection. Secreted $RT1A^a$, after pcRQ.B3 injection at the doses used in these experiments, was not detected in serum using an ELISA sensitive to 15 ng/ml (data not shown). However, indirect evidence for expression of the protein was indicated by: 1) priming of the humoral response to $RT1A^a$, and 2) consistent 1-day acceleration of ACI heart allograft rejection (1-mg dose). The membrane-bound or secreted nature of the MHC class I Ag produced as a result of transfecting either pcRT.45 or pcRQ.B3 was confirmed in vitro. Transfection of cells with pcRT.45 resulted in the production of $RT1A^a$ that was detectable in association with the cell membrane, but not detectable as a secreted molecule. In contrast, pcRQ.B3-transfection resulted in

the production of secreted, but not membrane-bound, $RT1A^a$. It is noteworthy that a small amount of $RT1A^a$ was detectable in cell lysates of pcRQ.B3-transfected cells. This result most likely represented intracellular molecules that had not been exported to the cell surface, because $RT1A^a$ molecules were not detectable on the surface of pcRQ.B3-transfected cells by FACS analysis.

Direct muscle injection of cDNA encoding either membrane-bound or secreted MHC class I Ag changed the kinetics of heart allograft survival, as well as the antibody response. Injection of pcRT.45 resulted in acceleration of allograft rejection by $\geq$2 days, while pcRQ.B3 injection resulted in only a 1-day acceleration of graft rejection. Lower priming by pcRQ.B3 compared with pcRT.45 injection suggests that either secreted $RT1A^a$ encoded by pcRQ.B3 is less immunogenic, or pcRQ.B3 is not well expressed by muscle cells. However, our in vitro results showing that similar levels of $RT1A^a$ are produced by myoblasts transfected with either pcRQ.B3 or pcRT.45 tend to exclude the trivial explanation of our in vivo results being caused by a large difference in gene expression between the two plasmids. Interestingly, the humoral response was consistently primed by pcRT.45 injection, but priming was less consistent in pcRQ.B3-injected animals. In some rats pcRQ.B3 injection induced an accelerated antibody response equal to, or greater than, pcRT.45-injected animals. In others, the priming was weak, or not discernible from controls. It is possible that the differing immunologic responses to membrane-bound and secreted $RT1A^a$, or the variability observed with pcRQ.B3 injection was caused by a local concentration effect in vivo. Secreted $RT1A^a$ could be rapidly removed from the local environment, depending on local vascularization, whereas membrane-bound $RT1A^a$ would likely remain at a relatively high local concentration as a result of association with nonmobile cells.

The immunologic response to MHC class I Ag depends on the direct and indirect pathway of alloantigen presentation to T cells. With the direct pathway of Ag presentation in organ transplantation, recipient T cells respond directly to intact MHC class I molecules on the surface of allogeneic cells. T cells that recognize these molecules have a high frequency, and are a major factor mediating early allograft rejection (28–31). With the indirect pathway allogeneic MHC class I Ag is recognized in the form of polymorphic peptides presented in association with recipient MHC class II molecules (32). This is a pathway by which Th cells recognize allogeneic MHC class I Ag. Although Ag-activated Th cells secrete cytokines that induce proliferation and differentiation of antibody-producing B cells, the contribution of indirect Ag presentation to the graft rejection process is poorly understood. Based on the ability of pcRQ.B3 injection to prime the antibody response to $RT1A^a$, our data supports a potential role for the indirect pathway of MHC class I Ag presentation in mediating graft rejection. It is likely that the indirect pathway of Ag presentation was used with pcRQ.B3 injection, because $RT1A^a$ molecules encoded by the plasmid were secreted and not retained by the cell membrane, as demonstrated by ELISA and FACS analysis, respectively. In support of this theory, we have recently found that high doses of water-soluble recombinant human/mouse fusion proteins $HLA\text{-}A2/Q10^b$ and $HLA\text{-}B7/Q10^b$ stimulate IL-2 production by $CD4^+$ human T cells via an indirect pathway (33). It has also been reported previously that monovalent MHC class I molecules ($H\text{-}2D^d$) genetically engineered into secreted proteins using the mouse $Q10^b$-region do not stimulate $CD8^+$, $H\text{-}2D^d$-specific, T cells in vitro unless they are rendered polyvalent (34). Because pcRT.45 injection primed the CTL and antibody response against donor tissue, and accelerated allograft rejection, one interpretation of our results would be that polyvalent, membrane-bound, MHC class I leads to more efficient allosensitization by utilizing both pathways of Ag presentation. Transfected myocytes that express membrane-bound $RT1A^a$ could directly stimulate host CTL, and the membrane-bound form, shed by the myocytes, could be processed and presented by host APC to recipient Th cells via the indirect pathway of Ag presentation.

Interestingly, pcRT.45 and pcRQ.B3 plasmid injections had a different effect on the CTL response. Injections of pcRQ.B3 did not appreciably prime the CTL response to cardiac allografts, whereas pcRT.45 injection did prime CTL. Although myocytes are not known to possess costimulator activity, the greater CTL response and greater acceleration of graft rejection caused by pcRT.45 compared with pcRQ.B3 injection suggests that some form of the direct pathway (i.e., stimulation of CTL by myocyte-expressed membrane-bound $RT1A^a$) may operate in effecting sensitization. A study by Rogers and Mescher (35), which shows CTL can be primed without costimulation using cell-sized particles coated with MHC class I Ag, suggests myocyte costimulatory activity may not be necessary for CTL priming.

Our findings indicate that acceleration of graft rejection was not directly correlated with levels of specific antibody because pcRQ.B3-injected rats consistently rejected allografts only 1 day early despite variable antibody priming. Thus, for example, a rat with a high IgG anti-ACI antibody response on day 3 rejected no faster than one with a low IgG antibody response. These results agree with previous studies in which cytotoxic antibody levels were not found to correlate with cardiac allograft rejection time (36, 37). Fangmann et al. (32) described the ability of the indirect pathway of allorecognition to sensitize high-responder strain Lewis rats. This group used two immunizations with CFA in combination with $RT1A^{avl}$-derived peptides to achieve accelerated rejection of a skin allograft and a secondary antibody response to $RT1A^{avl}$. Because CFA is lipophilic, this protocol may be more analogous to our use of cDNA encoding membrane-bound $RT1A^a$, which is also lipophilic.

In summary, results from this study support the premise that membrane-bound and secreted MHC class I Ag have different immunogenic properties in vivo, and show that

direct gene transfer to skeletal muscle is an effective means by which alloimmunity to specific MHC proteins can be examined.

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